Development of a serological Luminex assay for Trichinella and Salmonella in swine

Van der Wal FJ* [1],
Achterberg RP[1], Maassen CBM[1] [2]

Central Veterinary Institute of Wageningen UR, Department of Infection Biology, Lelystad, The Netherlands
(1) Central Veterinary Institute, part of Wageningen UR, P.O.Box 65, 8200 AB Lelystad, NL

[2] current address: National Institute for Public Health and the Environment (RIVM), Bilthoven, NL

*corresponding author : fimme.vanderwal@wur.nl

Abstract

In order to develop veterinary serological multiplex assays, a singleplex bead-based array on the Luminex platform was developed, and with this experience the study was expanded by building a multiplex serological assay. First a serological Luminex assay was developed for Trichinella in swine. As the developed assay performed comparable to commercial ELISA's, work on this platform was continued by developing a serological multiplex assay for Salmonella in swine. This assay is based on five LPS variants of the most important serogroups occurring in pigs. The serological multiplex assay for Salmonella performed comparable to a commercial ELISA. The results from this study demonstrate the feasibility of the Luminex platform for multiplex serology. Ultimately, this type of assay can be used for routine screening of porcine serum samples for immune responses against multiple pathogens in one assay.

Introduction

To combat infections and increase food safety, slaughter pigs are monitored for the presence of pathogens, amongst others zoonotic pathogens like Trichinella and Salmonella. Here we describe the development of serological suspension arrays for Trichinella and Salmonella in swine on the Luminex platform.

Trichinellosis is a parasitic zoonosis affecting at least 11 million people all over the world (1). The disease is caused by nematodes of the genus Trichinella, in humans most prevalently Trichinella spiralis, by ingestion of raw or undercooked meat. As trichinellosis is an OIE notifiable disease, in the European Union meat from over 167 million pigs is inspected each year (2) with an artificial digestion method using pooled material. For positive pools individual samples are investigated microscopically, altogether a labour-intensive and costly procedure. In 2006 the EU approved new legislation on risk-based Trichinella control using serology (SANCO/2537/2005); ELISA based on excretory/secretory (E/S) antigens of T. spiralis larvae has been described (3) but is not common practice in Europe.

Salmonella, the second zoonotic pathogen that is subject of this study, is considered a major public health hazard with more than 29.000 cases of gastroenteritis in the Netherlands alone. About 26% of these cases is attributed to the consumption of pork (4). In contrast to serology for Trichinella monitoring, serology is a widely accepted tool for Salmonella monitoring. In countries like Denmark and the Netherlands ELISA-based serology is used for monitoring the Salmonella status of pig herds (5). Commonly the major antigenic component lipopolysaccharide (LPS) is used for Salmonella serology, and various ELISA's are commercially available.

The Luminex technology enables simultaneous testing of multiple serological components within one sample, i.e. multiplex ELISA. The technology is based on flow cytometry and uses polystyrene microspheres (beads), available in different colours. The bead surface is carboxylated to allow covalent coupling of compounds, i.e. antigens in case of a serological assay (Fig. 1). By using combinations of differently coloured beads, where each set carries a distinctive antigen, multiplex serology is possible. The Luminex platform has successfully been used for multiplexed serology in human serum (6) and veterinary assays that detect antibodies against multiple pathogens have now been published (7).

In order to develop a bead-based serological assay that can serve as a scaffold for expansion with similar assays, we first focused on developing a Luminex assay for Trichinella serology in swine. As an evaluation showed that this assay performed comparable to commercial ELISA's, we continued to develop an assay with multiple Salmonella antigens for serology in pig serum. This developed assay also performed as good as a commercial ELISA. These results showed that the developed assays adequately detect serum antibodies in pig serum, and demonstrate the feasibility of the Luminex platform for multiplex serology in veterinary applications.

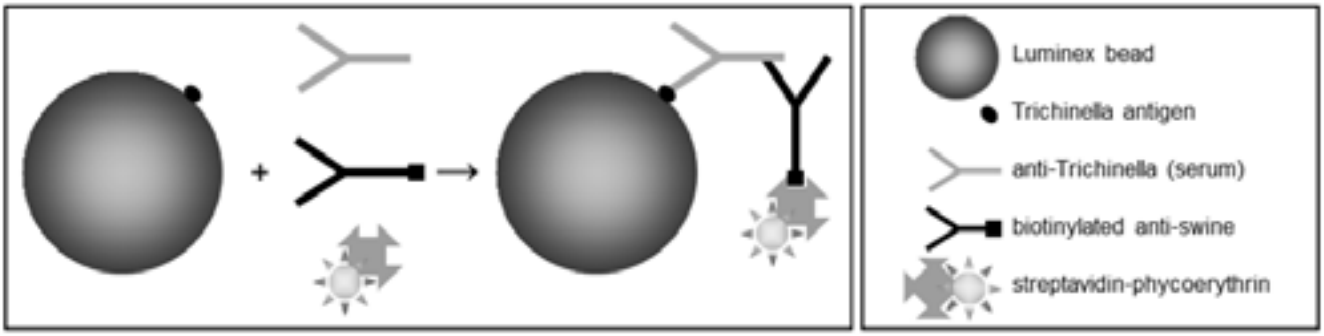

Figure 1 Principle of a serological Luminex assay. In this example Trichinella serology is depicted. A bead with covalently bound antigen is used to catch specific serum antibodies. These are sandwiched using biotinylated anti-swine antibodies and the complex is fluorescently labelled via biotin using streptavidin-phycoerythrin. With two lasers the Luminex system investigates the colour of beads (and thereby the bioassay) and the presence of fluorescent phycoerythrin, where the amount of fluorescence detected reflects the amount of antibodies caught from the serum sample.

Material and Methods

Trichinella excretory-secretory (E/S) antigens (J. van der Giessen, RIVM) and five Salmonella LPS's were purchased (Sigma) or isolated using standard protocols. Antigens were coupled to 2.5 x 106 carboxylated paramagnetic beads (Magplex microspheres, Luminex). Bead-based assays were performed with a flow cytometry-based Luminex 200, essentially as recommended by Luminex, using 1000 beads per assay. Assays were performed in phosphate buffered saline; for Salmonella assays a high salt concentration was used to reduce background. For antibody detection in pig and rabbit sera biotinylated anti-swine or anti-rabbit antibody (Jackson ImmunoResearch) was used in combination with streptavidin conjugated phycoerythrin (Invitrogen). Using xPONENT 3.0 software (Luminex) samples were analysed by measuring the fluorescence of minimal 100 beads per sample at default settings. Results were expressed as median fluorescence intensity (MFI). For evaluation purposes, results previously obtained with commercial ELISA's were used (two for Trichinella, one for Salmonella; C.B.M. Maassen, manuscripts in preparation). To compare results of suspension arrays with ELISA, diagnostic sensitivity (positives in test A / positives in B), diagnostic specificity (negatives in A / negatives in B), and overall agreement (positives and negatives in common / total number) are given. As a measure of agreement between assays Cohen's kappa was calculated using the online Graphpad Quickcalc software.

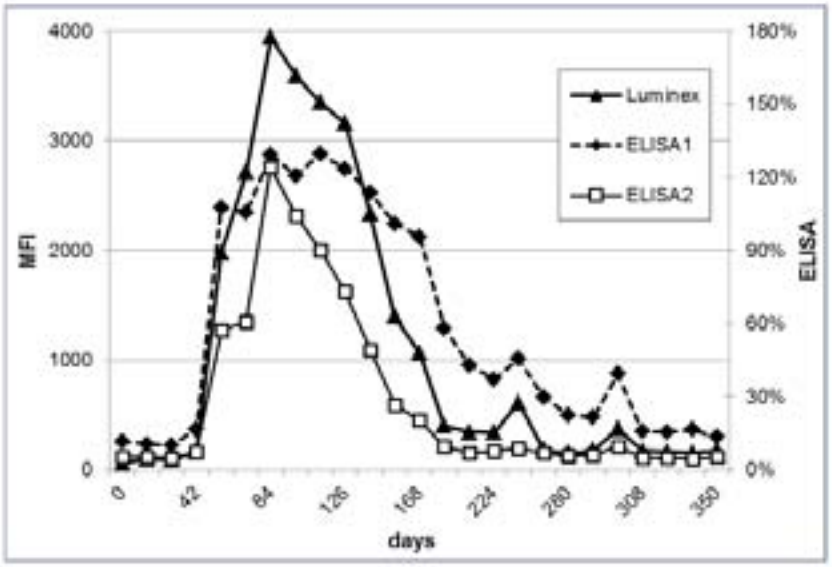

Figure 2 Comparison of the Luminex Trichinella assay with two ELISA's.
Longitudinal sera of an experimentally infected pig were tested with the Luminex assay (MFI, left y-axis) and ELISA (right y-axis). Results were plotted against days after infection.

Results

Longitudinal sera from an experimentally infected pig (R. Gamble) were tested with the Luminex Trichinella assay and the results were compared with results of two commercial ELISA's. With the Luminex assay an IgG response against Trichinella was detected that changed over time; the resulting curve was in essence similar to results obtained with commercial ELISA's (Fig. 2). For this animal, the Luminex assay even gave positive results at time points where the ELISA's had returned below their respective cut-offs. The Luminex assay was further evaluated by testing 150 porcine serum samples collected in Argentina were Trichinella is endemic. The Luminex assay showed an overall agreement of 93% with ELISA, with a Cohen's kappa of 0.82, demonstrating good agreement between the two assays. These results suggest that the developed Luminex assay is suitable for Trichinella serology.

		ELISA 1		
		pos	neg	total
Luminex	pos	39	7	46
	neg	4	100	104
	total	43	107	150
		se 91%	o.a. 93%	
		sp 93%	c.k. 0,82	

Table 1 Comparison of the Luminex Trichinella assay with ELISA. Field sera from an endemic area were tested in the Luminex assay using a cut-off of 1100 MFI. Results were compared with ELISA in a so-called 2x2 table. Diagnostic sensitivity (se) and specificity (sp), overall agreement (o.a.) and Cohen's kappa (c.k.) are given.

Using a similar approach a Salmonella fiveplex assay was developed with five LPS variants covering the most important Salmonella serogroups occurring in pigs (Table 2). The LPS beads were individually tested with O-antigen specific agglutination sera, routinely used for serotyping Salmonella isolates. Signals were generated on the beads in concordance with the LPS present (Fig. 3, cf. Table 2). Also in a multiplex set-up using 14 experimentally Salmonella-infected pigs the porcine sera reacted with the LPS beads in agreement with the serogroup of the strain used for infection (not shown).
Finally, the Luminex Salmonella fiveplex assay was evaluated by testing 150 porcine Dutch serum samples (Table 3). Using separate cut-offs for each bead (resp. 1700, 1500, 1500, 6000, and 4000 MFI), the Luminex assay showed an overall agreement of 91% with ELISA, with a Cohen's kappa of 0.82, demonstrating good agreement between the assays. These results suggest that the developed Salmonella fiveplex assay is suitable for Salmonella serology.

serogroup	subsp/serovar	O-antigens
B	S. Typhimurium	O4, O5, O12
C1	S. Choleraesuis	O6, O7
C1	S. Livingstone	O6, O7
C2	S. Newport	O6, O8
D	S. Enteritidis	O9, O12

Table 2 LPS used for the Luminex Salmonella assay. The O-antigens of each LPS used for coupling to Luminex beads are presented.

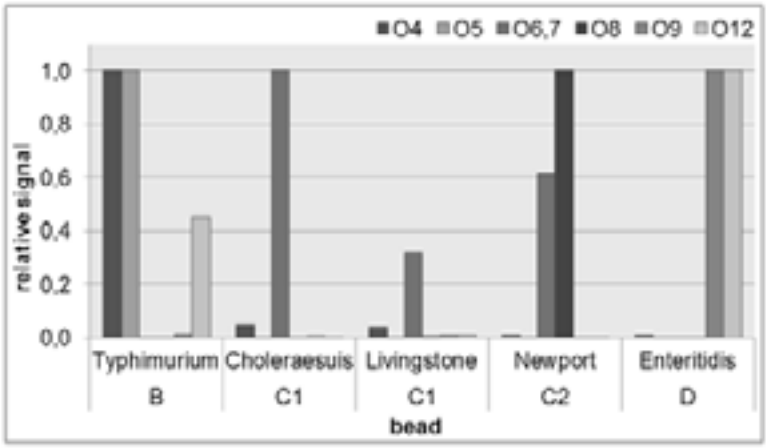

Figure 3 Luminex Salmonella LPS assay tested with agglutination sera.
Relative signal intensities (y-axis) are given for five Salmonella LPS beads tested with agglutination sera against O4, O5, O6/7, O8, O9 and O12. LPS donor strains are listed (x-axis).

		ELISA		
		pos	neg	total
Luminex	pos	54	7	61
	neg	6	83	89
	total	60	90	150
		se 90%	o.a. 91%	
		sp 92%	c.k. 0.82	

Table 3 Comparison of the Luminex Salmonella assay with ELISA. Dutch field sera were tested in the Luminex assay and compared with ELISA in a 2x2 table. For abbreviations see the legend to Table 1.

Discussion and Conclusion

During development of the serological Luminex assays many variables were tested (not shown) and it is possible that the assays presented here can be further improved. However, as these assays are meant as scaffold for expansion with similar assays, optimal conditions for the assays described here are not necessarily the optimal conditions for added tests; this would have to be evaluated upon each addition. In our experience for many variables a broad working range is possible. Variables that can have a profound effect on P/N-ratio (signal strength expressed as signal of a positive sample over signal of a well-defined negative sample (or pool of samples)) are sample dilution and salt concentration, and to a lesser extend incubation time, all very similar to ELISA. The assays presented here will have to be tested with large sample sets for validation before commercial application is possible. Topics that will need attention are the time required to perform assays, and the possibilities of automation to achieve the required sample throughput.
Early results showed that the Trichinella assay, developed to 'test drive' the Luminex platform, can successfully be added to the Salmonella fiveplex, even though initial assay conditions differ. We will use these assays as a scaffold for expansion with serological assays for zoonotic pathogens like Toxoplasma and pig pathogens like SVDV, Aujeszky disease virus and Mycobacterium avium subsp. avium.
Taken together the results show that the developed Trichinella and Salmonella assays can adequately detect antibodies in pig serum, thereby demonstrating the feasibility of the Luminex platform for multiplex serology in veterinary applications.

Acknowledgments

This research was financed by the Dutch Ministry of Economic Affairs, Agriculture and Innovation (Food Safety - Monitoring and detection KB-06-005).

References

1. Dupouy-Camet, J. 2000, Trichinellosis: a worldwide zoonosis, Vet Parasitol, 93: 191-200
2. Alban, L., et al. 2011, Towards a standardised surveillance for Trichinella in the European Union, Prev Vet Med, 99: 148-160
3. Gamble, H.R., et al. 1988, Evaluation of excretory-secretory antigens for the serodiagnosis of swine trichinellosis, Vet Parasitol, 30: 131-137
4. Aalten, M., et al. 2010, Staat van zoönosen 2009, RIVM report (Dutch)
5. Wegener, H.C., et al. 2003, Salmonella control programs in Denmark, Emerg Infect Dis, 9: 774-780
6. van Gageldonk, P.G., et al. 2008, Development and validation of a multiplex immunoassay for the simultaneous determination of serum antibodies to Bordetella pertussis, diphtheria and tetanus, J Immunol Methods, 335: 79-89
7. Anderson, S., et al. 2011, Development and evaluation of a Luminex multiplex serology assay to detect antibodies to bovine herpes virus, parainfluenza virus, bovine viral diarrhoea virus, and bovine respiratory syncytial virus, with comparison to existing ELISA detection methods, J Immunol Methods, 366: 79-88